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SALIVARY GLAND EXTRACTS OF *CULICOIDES SONORENSIS* INHIBIT MURINE LYMPHOCYTE PROLIFERATION AND NO PRODUCTION BY MACROPHAGES

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Abstract

Culicoides biting midges serve as vectors of pathogens affecting humans and domestic animals. *Culicoides sonorensis* is a vector of several arboviruses in North American that cause substantial economic losses to the US livestock industry. Previous studies showed that *C. sonorensis* saliva, like the saliva of many hematophagous arthropods, contains numerous pharmacological agents that affect hemostasis and early events in the inflammatory response, which may enhance the infectivity of *Culicoides*-borne pathogens. This paper reports on the immunomodulatory properties of *C. sonorensis* salivary gland extracts on murine immune cells and discusses the possible immunomodulatory role of *C. sonorensis* saliva in vesicular stomatitis virus infection of vertebrate hosts. Splenocytes treated with *C. sonorensis* mitogens were significantly affected in their proliferative response, and peritoneal macrophages secreted significantly less NO. A 66-kDa glycoprotein was purified from *C. sonorensis* salivary gland extract, which may be in part responsible for these observations and may be considered as a vaccine candidate.

INTRODUCTION

Several species of biting midges (Diptera: Ceratopogonidae) serve as vectors of diseases affecting humans and domestic animals. *Culicoides paraensis* is the primary vector of Oropouche virus to humans in Neotropical urban cycles.[1] *Culicoides sonorensis* is the primary vector of several economically important livestock diseases in North America.[2,3]

Hemostasis, inflammation, and immunity are major obstacles that hematophagous arthropods must circumvent when taking a blood meal on the vertebrate host. These physiologic barriers

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are inactivated by bioactive substances of hematophagous arthropods that are inoculated into the host skin through saliva during blood feeding.[4] Hematophagous arthropods share the ability to modulate the host's blood flow and the immune response through the effect of bioactive salivary factors. Bioactive salivary factors have been identified for mosquitoes, sand flies, hard ticks, black flies triatomine bugs, and biting midges.[4–14] These factors have been shown to play active roles during transmission and pathogenesis in experimental models of arthropod-borne diseases caused by arboviruses, bacteria, and protozoans.[4]

Culicoides sonorensis is the primary vector of the blue-tongue viruses in the United States,[2] and it has been implicated in the transmission of vesicular stomatitis virus (VSV) in the southwestern United States.[15–18] In addition to antihe-mostatics,[19–21] preliminary findings indicated the presence of salivary immunomodulatory factors in *C. variipennis* (*C. sonorensis* sensu stricto).[22] The objective of this study was to confirm and evaluate in detail the effects of *C. sonorensis* salivary gland extracts on murine immune cells. Here, we report the anti-proliferative effects on B and T cells and the reduced NO production by macrophages associated with exposure to *C. sonorensis* salivary factors and discuss their potential role in arbovirus transmission.

MATERIALS AND METHODS

Mice

Young adult C57BL/6 mice 4–6 weeks of age were purchased from the National Cancer Institute (Bethesda, MD). The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals. All studies requiring mice were performed in accordance with protocols approved by the Animal Care and Use Committee at Colorado State University.

Culicoides sonorensis and salivary gland extracts. *Culicoides sonorensis* salivary glands were dissected from 2- to 4-day-old *C. sonorensis* adult females from colony AK[23] and reared under conditions described elsewhere.[23] Salivary glands were dissected, collected in phosphate-buffered saline (PBS) and stored at −70°C before use. The glands were lysed by freezing and thawing in PBS. A pair of female salivary glands averaged 0.21 μg of soluble protein.[21] This estimate was used to calculate the amount of salivary extract protein used in experiments.

T- and B-cell proliferation assays

Optimal concentrations for Concanavalin A (Con A) with T cells and lipopolysaccha-ride (LPS) with B cells and macrophages were determined. Spleen cell suspensions were prepared from C57BL/6 mice in Dulbecco minimal essential medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 5×10^{-5} mol/L 2-mer-captoethanol and 0.5% normal mouse serum.[24] Aliquots containing 2×10^5 cells were added to the wells of 96-well culture plates (Costar, Corning, NY). The cells were pre-incubated for 2 hours in medium (final volume 100 μL) with or without *C. sonorensis* salivary gland extract (SGE).

Cells were stimulated with the mitogens, either T-cell mitogen Con A (Miles Laboratories, Naperville, IL) or B-cell mitogen LPS (*Escherichia coli* 055:B55W; Difco Laboratories, Detroit, MI), for 18 hours at specific concentrations. The degree of proliferation was determined by pulsing cultures with 1 μCi/well of ^{3}H-thymidine (Amersham, Arlington Heights, IL) for 8 hours followed by scintillation counting.[24]

To determine that there was no effect by SGE on the baseline proliferation of murine splenocytes, splenocytes were placed into culture with and without *C. sonorensis* SGE, and ^{3}H-thymidine was added at 16, 24, or 48 hours of culture to determine the degree of proliferation of the cells.

NO assays

Peritoneal exudate cells (PECs) were obtained from C57BL/6 mice and plated at 1×10^6/mL in 96-well culture plates.[25] After 2-hour incubation, non-adherent cells were removed by rinsing with medium, and the adherent cells, which were 95% macrophages, were incubated for 2 hours with medium or medium plus *C. sonorensis* SGE.[25,26] LPS was added to a final concentration of 10 ng/mL, and nitrite production (which reflects NO production by macrophages) was assessed at 72 hours thereafter by the Griess reaction.[24]

Lectin affinity chromatography

Purified salivary glycoproteins from *C. sonorensis* SGE were obtained from the PBS extract from 76 pairs of glands passed through a PBS-equilibrated 0.1-mL column of Con A immobilized on aga-rose beads (Vector Laboratories, Burlingame, CA). After incubation on ice for 1 hour, the flow through was collected, and the beads were washed three times with PBS before eluting the glycoproteins with 200 mmol/L methyl α-D-mannopyranoside (αMMP) in PBS. The eluate was processed for lectin-blot analysis and the B-cell proliferation assay. The B-cell assay was chosen to test glycoprotein fractions of *C. sonorensis* SGE because 1) Con A, a known T-cell mitogen, present in the eluate would stimulate the proliferation of T cells, and 2) B cells showed the greatest degree of proliferative inhibition. Therefore, CBP66 binding to Con A would not interfere with interpretation of results in the B-cell assay.

Lectin blot analysis

The electrophoretic separation of the fractions was conducted under reducing conditions on precast 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Invitrogen, Carlsbad, CA) in MOPS buffer, at 200 V for 50 minutes. Pre-stained rainbow protein mix (Amersham Biosciences, Piscataway, NJ) was used as molecular weight standards. The proteins were electrotransferred to nitrocellulose paper (Biorad, Hercules, CA) in tris-HCl glycine-methanol buffer at 100 V for 1 hour and stained with reversible stain MemCode (Pierce, Rockford, IL). After verifying protein transfer with MemCode, the membrane was destained and blocked with 1% bovine skin gelatin (Sigma, St. Louis, MO) in PBS. The membrane was incubated with biotinylated lectin (Vector, Burlingame, CA) diluted at 1 μg/mL in TST (0.05 mol/L Tris, pH 7.5, 0.15 mol/L sodium chloride, 0.1% Tween 20) for 1 hour at room temperature on a rocking platform. After washing three times with TST buffer, the membrane was incubated with neutra-vidin (Vector) 1 μg/mL and biotinylated alkaline phosphatase (Vector) 1 μg/mL, for 1 hour at room temperature. The membrane was incubated in BCIP/NBT (KPL, Gaithersburg, MD) enzyme substrate after washing three times with TST buffer. For the purpose of comparison, the equivalent of 10 pairs of *C. sonorensis* salivary glands was loaded on a 4–12% SDS-PAGE gel, and the proteins were separated as above and visualized using silver stain.

Statistical analyses

Data were analyzed using a Student *t* test, and differences were considered significant when $P < 0.05$ (SigmaPlot 9.0, Point Richmond, CA).

RESULTS

Inhibition of the Con A–driven mitogenic response by murine splenic T cells

Figure 1 shows that pre-incubation with *C. sonorensis* medium containing SGE significantly inhibited the proliferation of splenic T cells compared with cells pre-incubated in medium alone. Cells stimulated with 0.5 and 1.0 μg Con A/mL showed 66% inhibition ($P < 0.00001$) and 40% inhibition ($P < 0.001$), respectively.

Inhibition of the LPS-driven mitogenic response by murine splenic B cells

Figure 2 shows that *C. sonorensis* SGE significantly inhibited the polyclonal response of B cells. Cells stimulated with 10 μg LPS/mL plus 2 μg SGE showed 42% inhibition ($P < 0.01$), and 76% inhibition ($P < 0.00001$) was seen when the cells were stimulated with 30 μg LPS/mL plus 2 μg SGE.

Inhibition of NO production by murine macrophages

Peritoneal macrophages were pre-treated with *C. sonorensis* SGE for 2 hours before stimulation with LPS 10 ng/mL for 72 hours to induce the production of NO. Figure 3 shows that treatment with *C. sonorensis* SGE reduced the amount of NO secreted by LPS-stimulated macrophages by 59% ($P < 0.03$).

Lack of effect on the baseline proliferation of murine sple-nocytes

The immunoinhibitory effects of *C. sonorensis* SGE was not caused by a non-specific anti-proliferative effect of the extract on target cells (Figure 3). The viability of extract-treated cells did not differ significantly from control cells as assessed by trypan blue exclusion. The results shown in Figure 4 show that there is no significant anti-proliferative effect of SGE on murine splenocytes. The cells most likely to proliferate in response to LPS stimulation are B cells. By 48 hours of culture, there was a modest augmentation of proliferation at the higher dose of 2 μg SGE. These results suggest that a non-specific anti-proliferative effect was not responsible for the inhibitory effects that *C. sonorensis* SGE had on the functions of murine splenocytes.

A 66-kDa glycoprotein in the Con A–binding fraction retains inhibitory activity

Lectin affinity chromatography selectively purified glycoproteins present in the SGE that interact with Con A. Figure 5B shows a major 66-kDa Con A–binding protein (CBP66) eluted from the column. Incubation of murine splenocytes with dilutions of the CBP fraction, derived from the PBS extract of *C. sonorensis* salivary glands, inhibited the proliferation of splenocytes induced by LPS as indicated by a decrease in the incorporation of ^{3}H-thymidine (Figure 6). An identical affinity chromatography column was processed in parallel with only PBS in place of SGE as a negative control (data not shown). The 1:10 and 1:50 dilutions of CBP66 fractions significantly inhibited the proliferation of spleen cells by 71% ($P = 0.028$) and 75% ($P = 0.037$), respectively. A protein profile of *C. sonorensis* salivary glands in Figure 5A is shown for comparison. Approximately 20 silver-stained proteins are observed in this extract.

DISCUSSION

The *in vitro* data reported here extend previous observations from a variety of hematophagous arthropods showing that salivary gland extracts modulate the immune response of the vertebrate host. Two levels of normal immune cell function were negatively affected by *Culicoides* extracts. The expansion of both T and B mitogen-stimulated splenocytes was inhibited. These cells are capable of mounting a specific immune response to antigens/pathogens by the production of secreted products such as antibody by B cells. Second, macrophages were inhibited in their ability to produce NO in response to LPS. Macrophages can act as antigen-presenting cells and can be critical for the activation of T cells. In addition, they are phagocytic and are involved in killing intracellular pathogens including viruses, bacteria, and protozoans. One of the mechanisms by which macrophages kill intracellular *L. major* parasites is through the production of NO.[27–29] Moreover, NO has an effect on VSV infection *in vivo* that is mediated in the murine central nervous system by interleukin (IL)-12, interferon (IFN)γ, and NO synthase.[17,18] These observations and the results reported in this study are consistent with evidence that *Culicoides* saliva may have play a role in the establishment of VSV infection in vertebrate hosts.[11] The observations that *C. sonorensis*

transmission of VSV to cattle and guinea pigs is more efficient than direct syringe inoculation of higher titer inoculum support the hypothesis that *C. sonorensis* saliva increases the likelihood of VSV infection in susceptible vertebrate hosts.[17,18] The influence of *Culicoides* saliva in arbovirus infection requires further study.

Salivary gland components can be used as protective immunogens as shown in animal models.[12,30,31] It should be noted that chronic exposure of vertebrate hosts to vector saliva in endemic areas might be one of the factors involved in their age-related resistance to arthropod-borne diseases.[32,33] This is an intriguing observation; however, immunization with arthropod saliva or SGE is not a viable option. Therefore, specific molecules of the *C. sonorensis* salivary gland need to be purified for consideration as immunogens for a vaccine.

There are other abundant components derived from epithelial cells that are secreted with the saliva, and these can have profound effects on the immune system.[34–36] Cytoplasmic components not destined for secretion are not covalently modified with the N-linked glycans that are synthesized in the Golgi system. In arthropods, most N-linked glycans are of the paucimannosidic structure, which can be recognized by man-nose-specific lectins such as Con A.[37] Therefore, it is possible to affinity purify *C. sonorensis* SGE inhibitors using Con A, which recognizes mannose-rich N-linked glycans. Interfering with salivary immunomodulators might reduce the establishment of infection in vertebrate hosts. CBP66 is likely present in the saliva of *C. sonorensis*. Therefore, it could be a candidate antigen to control some of the microorganisms that *C. sonorensis* transmits to its vertebrate hosts. Furthermore, given the immunogenicity and antigenicity of arthropod glycans, the N-linked glycan attached to CBP66 may be the target of a vaccine.[38] Our research on *C. sonorensis* salivary gland pharmacology provides the foundation to explore the role of salivary factors in *Culicoides* vectors of animal diseases.

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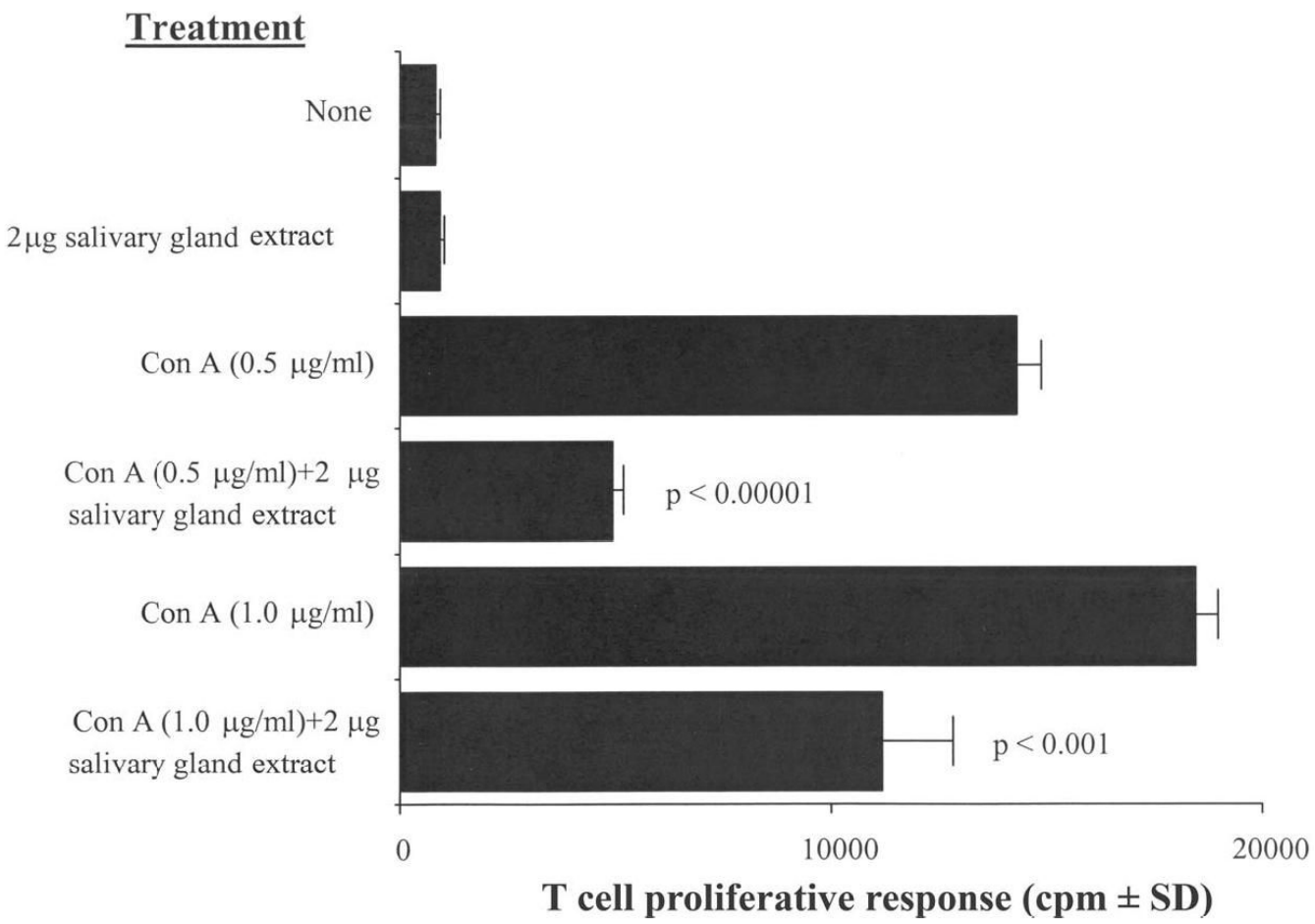

Figure 1.

Culicoides sonorensis salivary gland extract inhibits the Con A–driven mitogenic response of murine splenic T cells. C57BL/6 spleen cells were pre-incubated with 2 μg of *C. sonorensis* salivary gland extract for 2 hours and were stimulated with the indicated concentrations of Con A for 18 hours. The degree of proliferation by the splenocytes was assessed by scintillation counting. Salivary gland extract significantly inhibited proliferation; *P* values are indicated in the figure. The figure is representative of four independent experiments.

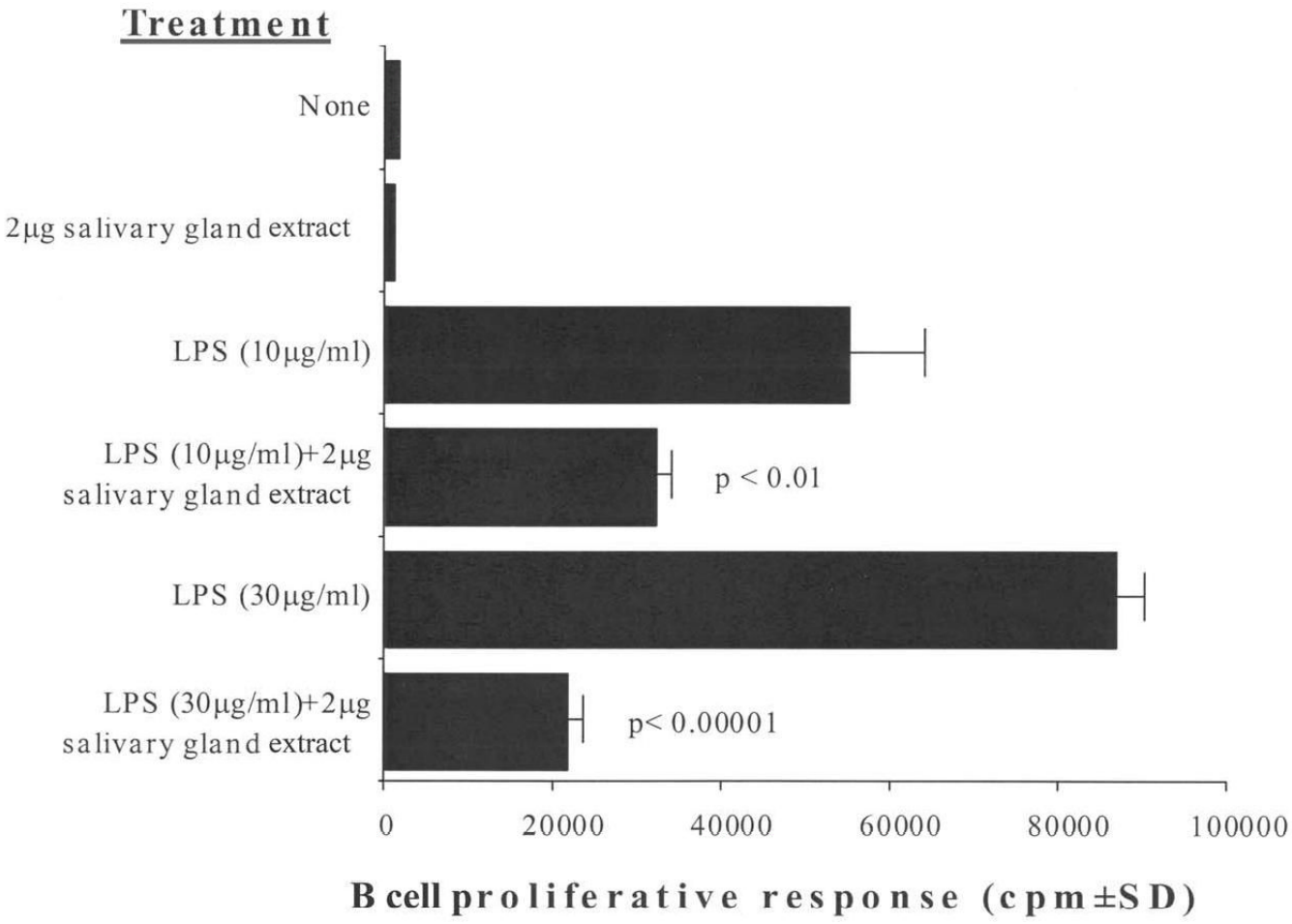

Figure 2.

Culicoides sonorensis salivary gland extract inhibits the LPS-driven mitogenic response of murine splenic B cells. C57BL/6 spleen cells were pre-incubated with 2 μg of *C. sonorensis* salivary gland extract for 2 hours and were stimulated with the indicated concentrations of LPS for 18 hours. Proliferation was assessed as in Figure 1. Salivary gland extract significantly inhibited proliferation; *P* values are indicated in the figure. The figure is representative of four independent experiments.

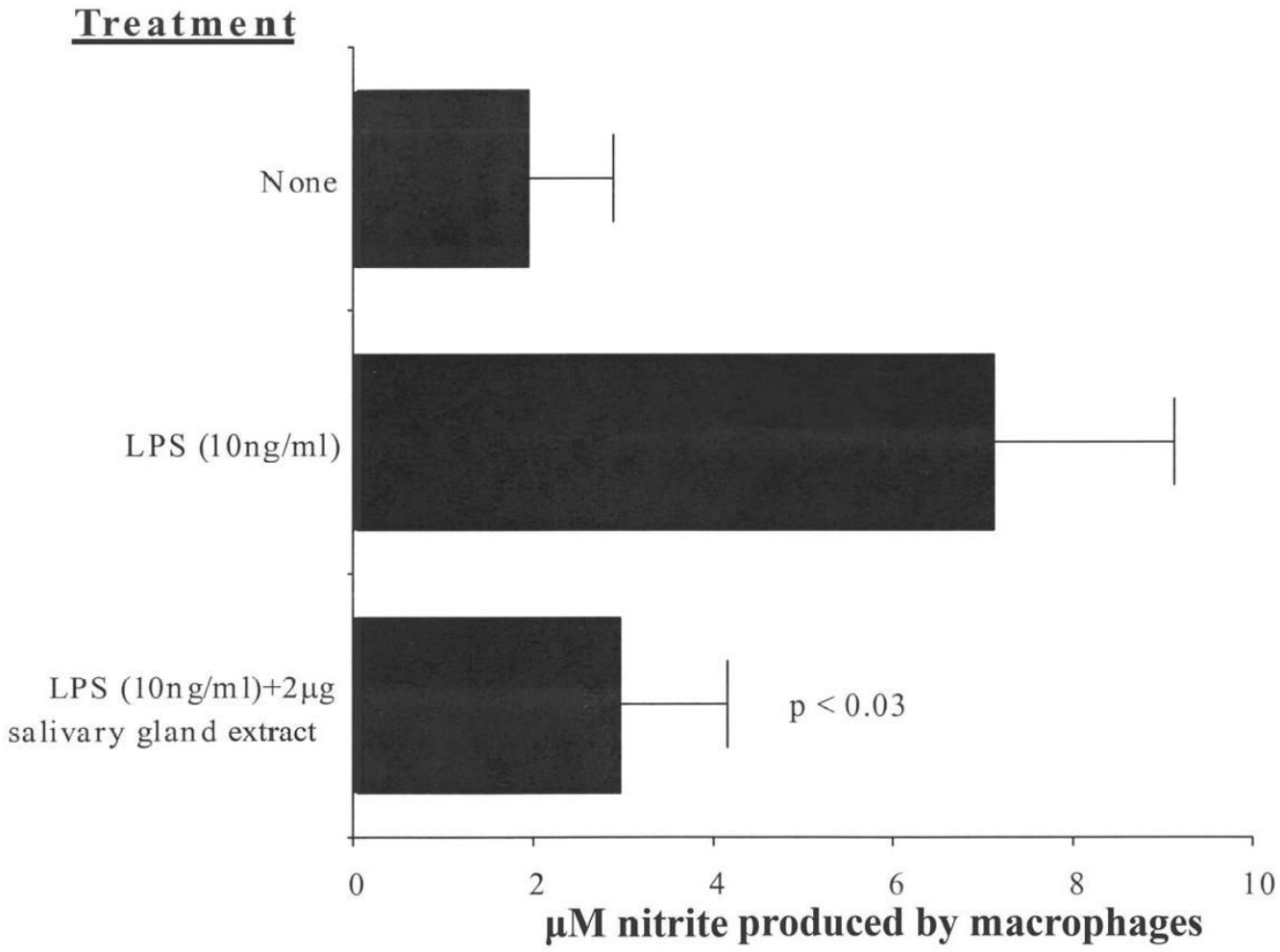

Figure 3.

Culicoides sonorensis salivary gland extract inhibits NO production by murine macrophages. C57BL/6 macrophages were pre-incubated with 2 µg of *C. sonorensis* salivary gland extract for 2 hours and were stimulated with 10 ng/mL of LPS for 72 hours. Nitrite production by the cells was assessed. Salivary gland extract significantly inhibited nitrite production; *P* values are indicated in the figure. The figure is representative of six independent experiments.

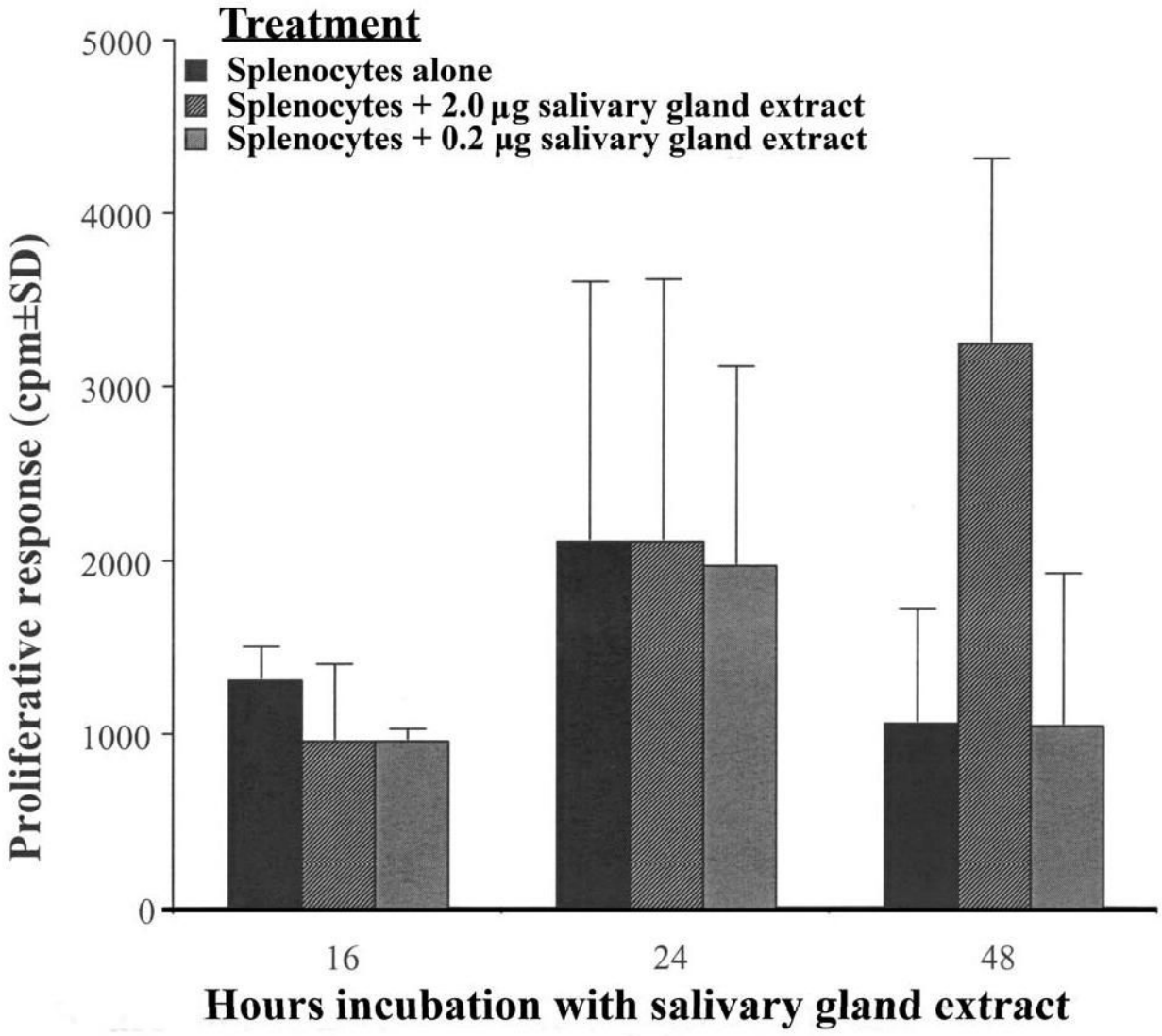

Figure 4.

Culicoides sonorensis salivary gland protein is not directly toxic for murine splenocytes. C57BL/6 spleen cells were incubated with the indicated amounts of *C. sonorensis* salivary gland protein for 16, 24, or 48 hours and were assessed for proliferation as in Figure 1. The figure is representative of two independent experiments.

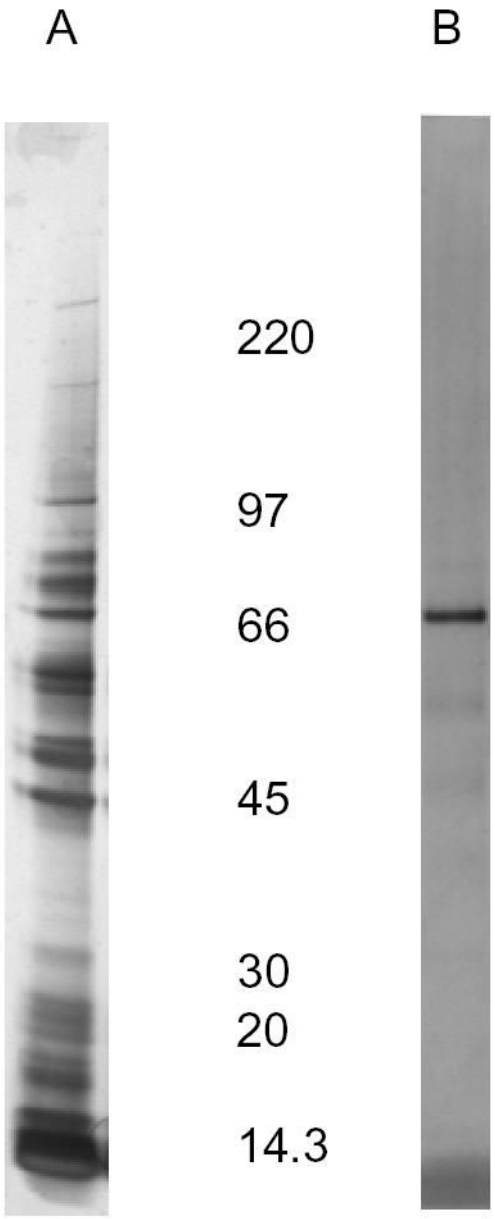

Figure 5.

Culicoides sonorensis Con A binding protein and SGE. (**A**), The equivalent of 10 pairs of salivary glands (2 μg) was loaded on a reducing SDS-PAGE gel, and the proteins were stained with silver stain. (**B**), Con A–binding protein of 66 kDa, eluted from a lectin affinity chromatography column, reacts with biotinylated Con A on a lectin blot. An identical affinity chromatography column was processed in parallel with only PBS in place of SGE as a negative control (data not shown). (**B**) is representative of two independent experiments.

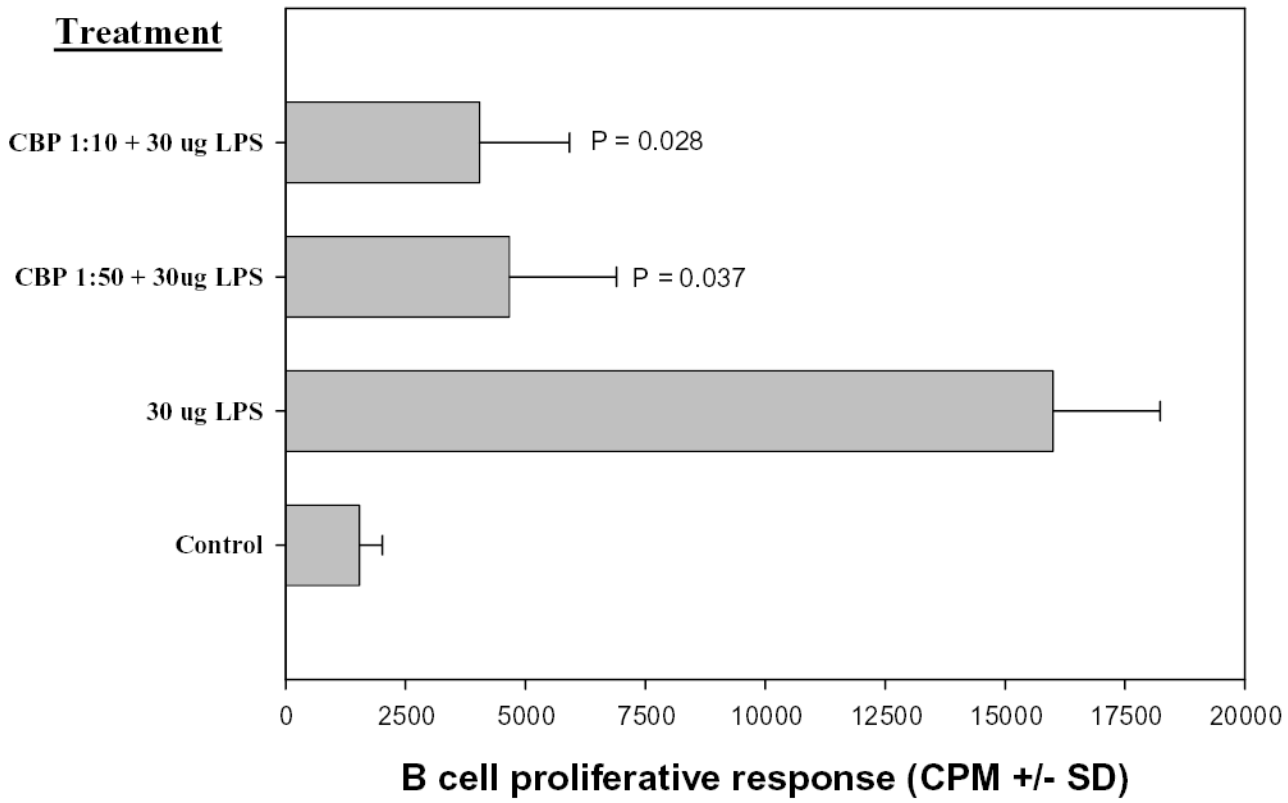

Figure 6.

Culicoides sonorensis CBP fraction inhibits proliferation of C57BL/6 splenic B cells. C57BL/6 spleen cells were treated with dilutions of CBP (1:10 or 1:50). After 2 hours, LPS was added to all wells with the exception of the negative control. Proliferation was assessed as in Figure 1. Both dilutions of CBP significantly inhibited proliferation. (CBP 1:10, $P = 0.028$; CBP 1:50, $P = 0.037$). This figure is representative of two independent experiments.